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## **Social behavior drives the dynamics of respiratory disease in threatened tortoises**

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## Social behavior drives the dynamics of respiratory disease in threatened tortoises

LORI D. WENDLAND,<sup>1,7</sup> JOHN WOODING,<sup>2</sup> C. LEANN WHITE,<sup>1</sup> DINA DEMCOVITZ,<sup>1</sup> RAMON LITTELL,<sup>3</sup> JOAN DIEMER BERISH,<sup>4</sup> ARPAT OZGUL,<sup>5</sup> MADAN K. OLI,<sup>5</sup> PAUL A. KLEIN,<sup>6</sup> MARY C. CHRISTMAN,<sup>3</sup> AND MARY B. BROWN<sup>1</sup>

<sup>1</sup>Department of Infectious Diseases and Pathology, College of Veterinary Medicine, University of Florida, Gainesville, Florida 32611 USA

<sup>2</sup>Consulting Wildlife Biologist, High Springs, Florida 32643 USA

<sup>3</sup>Department of Statistics, Institute of Food and Agricultural Sciences, University of Florida, Gainesville, Florida 32611 USA

<sup>4</sup>Florida Fish and Wildlife Conservation Commission, Gainesville, Florida 32663 USA

<sup>5</sup>Department of Wildlife Ecology and Conservation, University of Florida, Gainesville, Florida 32611 USA

<sup>6</sup>Department of Pathology, Immunology and Laboratory Medicine, College of Medicine, University of Florida, Gainesville, Florida 32611 USA

**Abstract.** Since the early 1990s, morbidity and mortality in tortoise populations have been associated with a transmissible, mycoplasmal upper respiratory tract disease (URTD). Although the etiology, transmission, and diagnosis of URTD have been extensively studied, little is known about the dynamics of disease transmission in free-ranging tortoise populations. To understand the transmission dynamics of *Mycoplasma agassizii*, the primary etiological agent of URTD in wild tortoise populations, we studied 11 populations of free-ranging gopher tortoises (*Gopherus polyphemus*;  $n = 1667$  individuals) over five years and determined their exposure to the pathogen by serology, by clinical signs, and by detection of the pathogen in nasal lavages. Adults tortoises ( $n = 759$ ) were 11 times more likely to be seropositive than immature animals ( $n = 242$ ) (odds ratio = 10.6, 95% CI = 5.7–20,  $P < 0.0001$ ). Nasal discharge was observed in only 1.4% (4/296) of immature tortoises as compared with 8.6% (120/1399) of adult tortoises. Nasal lavages from all juvenile tortoises ( $n = 283$ ) were negative by PCR for mycoplasmal pathogens associated with URTD. We tested for spatial segregation among tortoise burrows by size class and found no consistent evidence of clustering of either juveniles or adults. We suggest that the social behavior of tortoises plays a critical role in the spread of URTD in wild populations, with immature tortoises having minimal interactions with adult tortoises, thereby limiting their exposure to the pathogen. These findings may have broader implications for modeling horizontally transmitted diseases in other species with limited parental care and emphasize the importance of incorporating animal behavior parameters into disease transmission studies to better characterize the host–pathogen dynamics.

**Key words:** disease transmission; *Gopherus agassizii*; *Mycoplasma agassizii*; reptile behavior; URTD.

### INTRODUCTION

Gopher tortoises (*Gopherus polyphemus*) are long-lived, burrowing turtles endemic to the southeastern United States (Auffenberg and Franz 1982), that play a critical ecological role because they construct and live in subterranean burrows used for shelter by over 300 other vertebrate and invertebrate species (Jackson and Milstrey 1989). The gopher tortoise is experiencing declines throughout its geographic range (Enge et al. 2006) and recently has been reclassified as threatened in Florida. Although habitat loss is one of the most important reasons for these declines, infectious diseases also have been identified as a potential threat to tortoises (Enge et al. 2006).

Since the early 1990s, morbidity and mortality in tortoise populations have been associated with a transmissible, mycoplasmal, upper respiratory tract disease (URTD) in both the gopher tortoise and the federally threatened desert tortoise (*G. agassizii*) (Jacobson et al. 1991, McLaughlin 1997). Although there may be many infectious agents that affect gopher tortoises, *Mycoplasma agassizii* and a second species, *M. testudineum*, are presently the only confirmed, independent etiologic agents of URTD in wild North American tortoises (Jacobson et al. 1991, Brown et al. 1994, 1999). URTD has contributed to specific population declines of both the gopher and desert tortoise (Jacobson et al. 1991, 1995), and has become one of the most extensively characterized infectious diseases in free-ranging reptiles. The horizontal mode of transmission by respiratory secretions, clinical presentation, and diagnostic assays for mycoplasmal URTD have been well-established through experimental studies (Brown et

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<sup>7</sup> E-mail: WendlandL@vetmed.ufl.edu

al. 1994, 1995, 1999, Wendland et al. 2007); however, little is known about the dynamics of disease transmission in free-ranging tortoise populations.

Environmental and demographic factors are widely recognized as significant influences on pathogen transmission dynamics, particularly in humans and domestic animal hosts. However, there are few long-term epidemiological studies that describe these effects in wildlife populations (O'Brien et al. 2002, Hosseini et al. 2004, Altizer et al. 2006), and even fewer empirical studies have been conducted in wildlife relating host behavior to disease dynamics (O'Brien et al. 2002, Behringer et al. 2006). The majority of these studies describe increases in pathogen transmission associated with seasonal aggregations of animals for foraging or reproductive behaviors (O'Brien et al. 2002, Hosseini et al. 2004). These aggregations typically result in an influx of new susceptible hosts into the population which subsequently may drive recurrent epizootics (Hosseini et al. 2004).

We conducted a five-year epizootiological study of mycoplasmal URTD in 11 gopher tortoise populations to determine exposure to the pathogen by serology, by detection of the pathogen in nasal lavages, and by presence of clinical signs. We used these data to evaluate for specific demographic and spatial effects on the distribution of URTD. We report how the social structure and behavior of the gopher tortoise, a long-lived reptilian host, influences pathogen transmission dynamics within natural populations and may actually reduce the risk of infection until tortoises reach reproductive age.

#### METHODS

**Study sites.**—Gopher tortoises were sampled annually from 11 demographically independent sites in northern and central Florida from 2002–2006. Sites included 10 public lands and one privately-owned property. Five study sites had permitted tortoise relocations performed 10 or more years prior to our study, one site had suspected, non-permitted tortoise dumping activities, and the remaining five sites were state-owned lands with no documented relocation events. Habitat quality and type varied substantially among the sites, ranging from open pastures to pine flatwoods and sandhill habitats with varying degrees of management.

**Burrow selection and trapping.**—A burrow census was performed within each study area annually. Burrows were located, geo-referenced, and the burrow width was measured at 50 cm deep to estimate size class distributions of tortoises, since burrow width closely approximates tortoise body size (Martin and Layne 1987). Seventy-five to 125 active burrows were selected for trapping on each site annually using a stratified sampling scheme to obtain a representative sample by tortoise body size. Tortoises were captured in pitfall traps set directly in front of burrow openings. Opportunistic captures were also made. The University of Florida Institutional Animal Care and Use Committee,

and Florida Fish and Wildlife Conservation Commission approved all protocols for animal use (research permit #WX02160d).

For all animals captured, tortoises were photo-documented, morphometric measurements were taken (McRae et al. 1981a), tortoises were permanently marked for identification (Cagle 1939), and blood and nasal lavage samples were collected. Because sexual maturity is achieved at different ages depending on the sex of the tortoise, the availability of food resources, and the geographic latitude of origin, carapace length (CL) was used to estimate maturity level of the tortoise (Landers et al. 1982). Sexual maturity was assumed to have occurred at  $\geq 180$  mm CL for males and  $\geq 220$  mm CL for females (Diemer and Moore 1994).

Comprehensive health assessments were performed on each tortoise captured, but only methods pertaining to the presence of nasal discharge are presented. In the field, nasal discharge was graded as absent (0), mild (1), moderate (2), or severe (3); however, for statistical analyses, presence/absence categorical variables were used.

**Biological sample collection.**—Approximately 0.25–3.0 mL of blood, not to exceed 10% of the tortoise's estimated blood volume, was collected from the brachial vein of each tortoise. The subcarapacial venous sinus was used for blood collection from hatchlings or as an alternative venipuncture site in adults. Blood samples were placed in lithium heparin Microtainer tubes (Becton Dickinson, Cockeysville, Maryland, USA) and plasma was transferred to polypropylene tubes and stored at  $-20^{\circ}\text{C}$  in a manual defrost freezer. Nasal lavages were performed on tortoises using from 0.5 mL (hatchling/juvenile) to 5 mL (mature adult) of sterile 0.9% sodium chloride solution flushed into each naris. To prevent aspiration or swallowing of the flush, pressure was applied to the soft tissue between the ventral mandibles, forcing the tortoise's tongue into the choanae. The flush was collected in a sterile plastic container, and SP4 medium (Tully et al. 1979) was immediately added to the nasal wash at a volume of 10% of the flush volume.

**Laboratory analyses.**—Antibody levels for *M. agassizii* were detected by ELISA (Wendland et al. 2007). Culture and PCR techniques on nasal flush samples were similar to methods previously described (Brown et al. 2001), except as follows. A 400- $\mu\text{L}$  aliquot of the nasal flush sample was inoculated into 4 mL of SP4 broth (Tully et al. 1979), 2 mL were filtered using a 0.45- $\mu\text{m}$  filter to remove fungal contaminants, and 20  $\mu\text{L}$  of each broth sample and the nasal lavage fluid were plated on SP4 agar. Broth cultures were analyzed for the presence of *M. agassizii* DNA based upon PCR amplification of a portion of the 16S rRNA gene (Brown et al. 1995). Restriction fragment length polymorphism (RFLP) analysis of the 16S rRNA gene was conducted using *Age*I or *Nci*I on all positive PCR

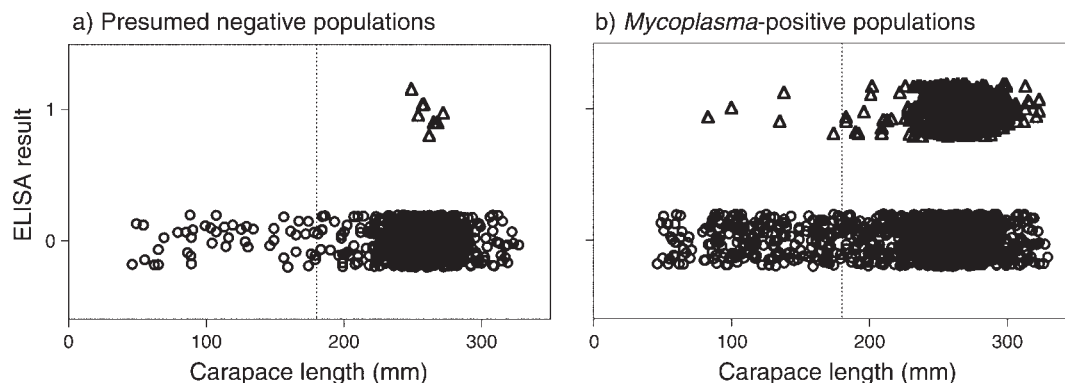


FIG. 1. Relationship between tortoise size and exposure to *Mycoplasma agassizii*. ELISA results from 1667 gopher tortoises plotted by carapace length (CL) in millimeters. Negative and suspect results were classified as 0, positive results were classified as 1, and a jitter function was applied to spread results along the y-axis. The dotted line represents the approximate onset of sexual maturity for tortoises. (a) Results from tortoise study populations that were classified as presumed *Mycoplasma*-negative based on serology, culture/polymerase chain reaction (PCR) analyses of nasal flushes (Brown et al. 1995), and presence of clinical signs of upper respiratory tract disease (URTD) among tortoises (four sites;  $n = 666$  tortoises). The eight seropositive results from these sites likely represent false-positive results given the predictive values of the assay at low prevalence (Wendland et al. 2007). (b) Results from *Mycoplasma*-positive populations (seven sites;  $n = 1001$  tortoises). Seropositive results were detected almost exclusively in tortoises with a CL  $> 180$  mm.

samples to confirm that the isolates were either *M. agassizii* or *M. testudineum* (Brown et al. 1995).

**Statistical analyses.**—Carapace length (CL) was used to establish the maturity level of tortoises, with sexual maturity assumed at  $\geq 180$  mm CL for males and  $\geq 220$  mm CL for females (Diemer and Moore 1994). Cumulative distribution functions were calculated for carapace length (CL) by ELISA and PCR results and for nasal discharge expression. The cumulative distribution function at any value of CL equals the proportion of subjects with a CL less than that value. The distributions for positive and negative results were then compared using Kolmogorov-Smirnov statistics for a one-sided test (Conover 1971). Associations between the odds of a tortoise having a positive ELISA result and the tortoise's sex and age class, (i.e., adult vs. immature), were evaluated using Cochran-Mantel-Haenszel statistics controlling for potential site effects (Woolson 1987).  $P$  values  $\leq 0.05$  were considered statistically significant for all analyses.

For testing of spatial clustering of juveniles and adults, the active burrow data from three years (2004–2006) were used from the 11 sites. Active burrows were categorized as either juvenile or adult based on width of the burrow entrance, with a burrow width  $> 220$  mm being labeled as an adult. Of the potential 33 data sets, 13 could not be used for testing since the data set either had insufficient numbers of juvenile burrows or no juvenile burrows for which the nearest neighbors were juveniles. Data sets were analyzed using a two-degrees-of-freedom contingency table analysis of spatial segregation (Dixon 1994) with expectations and variances based on complete mapping of the site. The null hypothesis was that the nearest neighbors of juveniles included juveniles and adults in proportions close to that expected under random labeling of burrows. Because the

data were from multiple years at the same sites, we used the Benjamini and Hochberg (Benjamini and Hochberg 1995) method to adjust  $P$  values to control the family-wise error rate.

## RESULTS

We determined exposure to *M. agassizii* among 1667 gopher tortoises from 11 study sites using ELISA (Wendland et al. 2007). The sex ratio of male to female tortoises sampled across the five-year period was 1.05:1 and therefore, approximately equal numbers of each sex were included in the analysis. Study sites were classified as mycoplasma-positive (seven sites;  $n = 1001$  tortoises) or presumed negative (four sites;  $n = 666$  tortoises) based on serology, culture/polymerase chain reaction (PCR) analyses of nasal flushes (Brown et al. 1995), and presence of clinical signs of URTD among tortoises. Male tortoises within the positive populations were more likely to be seropositive than female tortoises (odds ratio (OR) = 1.6, 95% CI = 1.1–2.1, groups significantly different at  $P = 0.005$ ). Additionally, seropositive results were detected almost exclusively in tortoises with a CL  $> 180$  mm (Fig. 1a, b). Tortoises classified as adults ( $n = 759$ ) were 11 times more likely to be seropositive than immature animals ( $n = 242$ ) within the positive populations (OR = 10.6, 95% CI = 5.7–20, groups significantly different at  $P < 0.0001$ ). Further, cumulative distribution functions were plotted for the carapace length of tortoises by ELISA result, and distributions for positive and negative ELISA results were found to be statistically different ( $P < 0.0001$ ; Fig. 2). The majority of immature tortoises with positive ELISA results (11/16) were between 181 and 220 mm in CL, and these tortoises likely were approaching sexual maturity. The five immature tortoises with CL  $< 180$



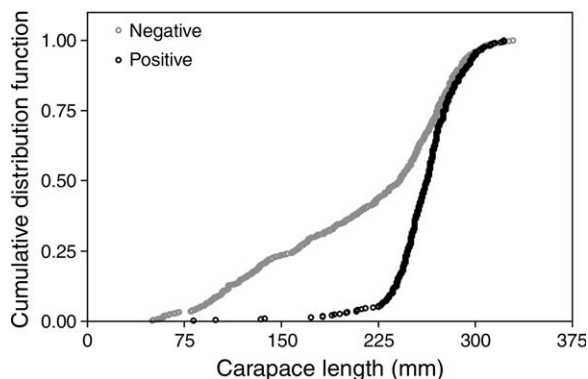


FIG. 2. Cumulative distribution functions plotted for the carapace length of tortoises by ELISA result. The cumulative distribution function at any value of CL equals the proportion of subjects with a CL less than that value. Distributions for positive (black circles) and negative (gray circles) ELISA results were found to be statistically different ( $P < 0.0001$ ), further demonstrating the pattern of positive ELISA results occurring primarily after tortoises reach mature size classes.

mm originated from two populations undergoing acute mycoplasmal epizootics.

Clinical sign expression data as well as culture and PCR analyses of nasal lavages were consistent with the ELISA findings. Nasal discharge, the most characteristic clinical sign of URTD (Brown et al. 1994, 1999), was exhibited predominantly by adult tortoises. Only 1.4% (4/296) of immature tortoises had a nasal discharge, whereas 10% (68/715) of adult males and 8% (52/684) of adult females exhibited a nasal discharge. Nasal lavages from all juvenile tortoises ( $n = 283$ ) were negative by PCR for *M. agassizii* and *M. testudineum*. Cumulative distribution functions were evaluated for CL of tortoises with positive and negative PCR and nasal discharge results; distributions for both differed significantly ( $P = 0.003$  and  $P < 0.0001$  for PCR and nasal discharge, respectively).

To determine if there were spatial barriers to interactions between adult and juvenile tortoises, we evaluated for spatial segregation among tortoise burrows by size class. Of the 20 data sets for which sufficient information was available (see *Methods*), only three had statistically significant clustering of the juveniles (adjusted  $P < 0.05$ ). Additional tests indicated this was exclusively due to juveniles being nearest neighbors of other juveniles more often than expected ( $P$  values ranged from  $<0.0001$  to  $0.016$ ), since tests of whether adults tended to show clustering were not significant for any of these sites (adjusted  $P > 0.05$ ). Our data sets came from 11 sites measured in three different years. Hence we also considered whether clustering of juveniles was ephemeral or persistent over the three years. We found that only one site had statistically significant segregation of juveniles in two of the three years analyzed indicating that segregation was not typical behavior in gopher tortoises in our study areas. The

remaining data sets had no evidence of clustering of either juveniles or adults.

## DISCUSSION

Previously, immature gopher tortoises were shown to be susceptible to infection with *M. agassizii* and demonstrated characteristic clinical signs, seroconversion, and presence of the organism within the nasal cavity (McLaughlin 1997). Gopher tortoises are colonial, and adult and immature burrows on the study sites often occur in close proximity to one another. Therefore, the observed relationship between sexual maturity and mycoplasma transmission dynamics was surprising. We considered the possibility that *M. agassizii*-induced mortality in immature animals was very high and occurred rapidly. However, carcasses from immature size classes were rarely observed during comprehensive burrow surveys. Only 7.5% (11/146) of the tortoise carcasses detected from 2002–2006 were from pre-reproductive size classes, including hatchling to subadult sized tortoises. Further, active burrows in the size range for immature tortoises were commonly observed in the positive populations, thus providing evidence of their presence.

Unlike mammalian and avian hosts, most reptiles have limited or no parental care of their young and close physical contact between adult and immature animals may only occur as immature animals approach sexual maturity. Thus, the transmission dynamics of mycoplasmal URTD may more closely resemble that of a sexually transmitted infection, rather than a respiratory disease transmitted via close proximity. This pattern appears to be unique among respiratory pathogens in general, but may be more common for infectious diseases in which the hosts have minimal direct contact until reaching sexual maturity (Altizer et al. 2003). In our study, significant evidence of spatial clustering among size groups was not observed supporting that the lack of mycoplasma transmission to juveniles likely represents a behavioral characteristic rather than a lack of opportunity for contact between the size classes. Thus, direct social interactions between juvenile and adult size classes of tortoises may be limited, and in most cases, insufficient for the transfer of infectious respiratory secretions. More substantial contacts, such as those that occur during courtship, mating, and agonistic behaviors, likely are required for transmission of the pathogen.

These findings have important implications for the modeling of transmission dynamics for this host-pathogen system. Density-dependent models are frequently used to characterize the transmission of pathogens that require direct contact for transfer (Ryder et al. 2005), whereas frequency-dependent models are often used to describe sexually transmitted infections (Lloyd-Smith et al. 2004). Population density likely has a significant influence on disease transmission among male tortoises, as males attain sexual maturity at a

smaller size, have larger home ranges (McRae et al. 1981b), and also participate in intraspecific aggression more commonly than do females (Auffenberg 1969). Male tortoises within the positive populations were slightly more likely to be seropositive than female tortoises (OR = 1.6, 95% CI = 1.1–2.1, groups significantly different at  $P = 0.005$ ). Further, a greater proportion of males seroconverted at a smaller size than did females (L. D. Wedland et al., *unpublished data*), suggesting that younger males may be particularly vulnerable as they attempt to establish their place in the social hierarchy. However, population density may be less important for disease transmission in female tortoises. Gopher tortoises exhibit a scramble-competition, polygynous mating system in natural populations (Boglioli et al. 2003), and mating opportunities for females may not be significantly affected by distance to the nearest neighbor (i.e., density; Boglioli et al. 2003). Certainly, a density threshold likely exists under which tortoise numbers are so low that mating opportunities will be limited. However, in populations with normal tortoise densities, the transmission dynamics for females may follow more of a frequency-dependent pattern, with the proportion of infected males driving transmission events. Further, in populations that consist of a high proportion of juveniles, overall transmission rates are more likely to be low. Our findings emphasize the importance of incorporating animal behavior parameters into mathematical models for disease transmission to better characterize the host-pathogen dynamics (Altizer et al. 2003).

Other horizontally transmitted pathogens of gopher tortoises that require prolonged contact for transfer of the microorganism may follow a similar pattern if behavior is driving these events. During mortality events caused by pathogens having minimal environmental transmission, immature size classes may be spared, providing a pool of tortoises for later recruitment. A significant limitation is that these size classes generally constitute a very small proportion of the overall population, and their numbers are usually inadequate to sustain a population. Managing upland habitats to increase the successful recruitment of juvenile tortoises may be a valuable conservation strategy in these cases. Alternatively, land managers could target smaller size classes for augmentation or restocking of depleted populations to reduce the risk of pathogen introduction. Certainly, such strategies will not be successful in the case of an infectious disease that can persist outside the host within the environment. Understanding disease transmission dynamics in the context of tortoise social behaviors will be an important consideration for the success of future conservation programs to ensure persistence of this keystone species.

Mass mortality events and local extirpations of imperiled herpetofauna have resulted in an increased recognition of the threat of infectious diseases to these species (Daszak et al. 1999, 2000, Pounds et al. 2006,

Smith et al. 2009). This study provides empirical evidence that reptiles likely have significant social behavior patterns that are under-appreciated and understudied, especially with regards to how these behaviors influence disease transmission. Behavior may substantively influence disease dynamics, even in evolutionarily conserved hosts that have traditionally not been thought to have complex social systems.

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